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ORIGINAL ARTICLE

Association of oestrogen receptor α gene polymorphisms with postmenopausal bone loss, bone mass, and quantitative ultrasound properties of bone

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Background: The gene encoding oestrogen receptor α (*ESR1*) appears to regulate bone mineral density (BMD) and other determinants of osteoporotic fracture risk.

Objective: To investigate the relation between common polymorphisms and haplotypes of the *ESR1* gene and osteoporosis related phenotypes in a population based cohort of 3054 Scottish women.

Results: There was a significant association between a common haplotype "px", defined by the *PvuII* and *XbaI* restriction fragment length polymorphisms within intron 1 of the *ESR1* gene, and femoral neck bone loss in postmenopausal women who had not received hormone replacement therapy ($n = 945$; $p = 0.009$). Annual rates of femoral neck bone loss were $\sim 14\%$ higher in subjects who carried one copy of px and 22% higher in those who carried two copies, compared with those who did not carry the px haplotype. The px haplotype was associated with lower femoral neck BMD in the postmenopausal women ($p = 0.02$), and with reduced calcaneal broadband ultrasound attenuation (BUA) values in the whole study population ($p = 0.005$). There was no association between a TA repeat polymorphism in the *ESR1* promoter and any phenotype studied, though on long range haplotype analysis subjects with a smaller number of TA repeats who also carried the px haplotype had reduced BUA values.

Conclusions: The *ESR1* px haplotype is associated with reduced hip BMD values and increased rates of femoral neck bone loss in postmenopausal women. An association with BUA may explain the fact that *ESR1* intron 1 alleles predict osteoporotic fractures by a mechanism partly independent of differences in BMD.

Osteoporosis is a common disease with a strong genetic component, characterised by low bone mineral density (BMD), microarchitectural deterioration of bone tissue, and an increased risk of fracture.¹ Although BMD is the major predictor of osteoporotic fracture risk,^{2,3} quantitative ultrasound (QUS) properties of bone have recently been found to predict osteoporotic fractures⁴ independent of BMD,^{5–7} showing that QUS provides information about osteoporotic fracture risk beyond that which can be obtained from BMD. This is thought to reflect the fact that QUS provides information on bone structure and quality that is distinct from BMD.⁸ Twin and family studies have shown that BMD and QUS (measured by broadband ultrasound attenuation and speed of sound) are under strong genetic control.^{9–14} The genes responsible for regulating these phenotypes are incompletely defined, but genetic linkage studies have identified several quantitative trait loci for the regulation of BMD^{15–19} and QUS.^{20,21} Moreover, polymorphisms in many candidate genes have been identified as determinants of BMD and include the vitamin D receptor,²² oestrogen receptor α (*ESR1*),^{23,24} *COL1A1*,²⁵ and *TNFRSF1B*,²⁶ although much less information is available on candidate genes for QUS regulation.

One of the most important and widely studied candidate genes for osteoporosis is *ESR1*. While many polymorphisms have been described in *ESR1*, the most widely studied are a dinucleotide repeat (TA) polymorphism located approximately 1.2 kb upstream from the first exon (dbSNP id = rs3138774) and two single nucleotide polymorphisms, located in the first intron, which are commonly detected by restriction fragment length polymorphism assays using the enzymes *PvuII* (rs2234693) and *XbaI* (rs9340799). Previous association studies of these polymorphisms in relation to

BMD have yielded inconsistent results, probably because of factors such as small sample size and differences between the study populations in terms of age, menopausal status, and ethnic background.^{23–25} Moreover, there is little information on the possible association between *ESR1* polymorphisms and QUS values, or on the relation with postmenopausal bone loss.^{29,36–39}

In order to clarify the role of *ESR1* as a candidate gene for osteoporosis related phenotypes, we have investigated three common polymorphisms and haplotypes of the *ESR1* gene in relation to BMD, bone loss, and QUS in a large population based study of 3054 women from the north east of Scotland. To our knowledge, this is the largest study investigating the association between *ESR1* gene polymorphisms and bone characteristics in women from an ethnically homogeneous population.

METHODS

Study subjects

The study group comprised white women who were randomly selected from the community health index, a database of all patients registered with a general practitioner from a 25 mile radius of Aberdeen, a city in the north east of Scotland, as part of a large prospective population based screening programme for osteoporotic fracture risk (the APOSS study).⁴⁰ The screening programme involved approximately 7000 women who were invited by letter to undergo

Abbreviations: APOSS, Aberdeen prospective osteoporosis screening study; BMD, bone mineral density; BMI, body mass index; BUA, broadband ultrasound attenuation; HRT, hormone replacement therapy; LRH, long range haplotype; QUS, quantitative ultrasound; SNP, single nucleotide polymorphism

Table 1 Characteristics of the study subject at baseline and follow up according to menopausal status and use of hormone replacement therapy

| Characteristic | Baseline | Follow up | | | |
|--------------------------------------|------------------------|------------------------|------------------------|--------------------------|-------------------------|
| | Total study population | Total study population | Postmenopausal, no HRT | Postmenopausal HRT users | Pre- and perimenopausal |
| Number | 3054 | 3054 | 945 (30.9%) | 1802 (59.0%) | 307 (10.1%) |
| Age (years) | 48.5 (2.3) | 54.7 (2.2) | 55.0 (2.2) | 54.8 (2.2) | 53.0 (1.5) |
| Body mass index (kg/m ²) | 25.3 (4.4) | 26.5 (4.7) | 26.8 (4.8) | 26.3 (4.6) | 27.0 (5.0) |
| LS BMD (g/cm ²) | 1.051 (0.160) | 1.009 (0.168) | 0.990 (0.160) | 1.004 (0.166) | 1.099 (0.175) |
| FN BMD (g/cm ²) | 0.880 (0.125) | 0.837 (0.123) | 0.831 (0.120) | 0.832 (0.122) | 0.886 (0.124) |
| Changes in LS BMD (%/year) | – | –0.614 (1.223) | –1.337 (1.048) | –0.322 (1.186) | –0.084 (0.932) |
| Changes in FN BMD (%/year) | – | –0.745 (1.059) | –1.210 (1.016) | –0.558 (1.020) | –0.401 (0.945) |
| Calcaneal BUA (dB/MHz)* | – | 75.5 (17.0) | 73.2 (17.3) | 76.1 (16.8) | 79.0 (16.4) |
| Calcaneal SOS (m/s)* | – | 1553.1 (32.8) | 1548.2 (31.2) | 1554.1 (32.7) | 1561.5 (34.5) |
| Time since menopause (years) | 2.3 (4.5) | 5.7 (6.3) | 6.3 (5.0) | 6.3 (6.9) | – |
| Follow up time (years) | – | 6.2 (0.7) | 6.2 (0.7) | 6.3 (0.8) | 6.1 (0.7) |

Data are mean (SD).

*Data for broadband ultrasound attenuation (BUA) and speed of sound (SOS) at the calcaneus were available for 2449 subjects at follow up visit (742 postmenopausal women who were not HRT users, 1465 HRT users, and 242 pre- and perimenopausal women).

BMD, bone mineral density; BUA, broadband ultrasound attenuation; FN, femoral neck; HRT, hormone replacement therapy; LS, lumbar spine; SOS, speed of sound.

bone mineral density (BMD) measurements between 1990 and 1994. In all, 5119 women (~73%) attended for evaluation and underwent BMD measurement at the lumbar spine and femoral neck and completed a risk factor questionnaire. This included details of fracture history, menopausal status, and use of hormone replacement therapy (HRT). Participants were weighed on a set of balance scales calibrated to 0.05 kg (Seca, Hamburg, Germany) and height was measured using a stadiometer (Holtain, Crymych, Dyfed, UK). All women were invited to undergo further assessment between 1997 and 1999, and 3882 women (75.8%) re-attended for evaluation. Baseline investigations were repeated at the follow up visit and information was gathered on fracture history by completion of a questionnaire. Calcaneal broadband ultrasound attenuation and speed of sound was measured and blood samples were obtained for DNA extraction from 3054 women who consented. Women were classified as premenopausal if they were menstruating regularly, as perimenopausal if menstruation was irregular and/or up to six months had elapsed since their last period, and postmenopausal if menstruation had ceased for six months or more. Information gathered on menopausal status and HRT use identified five categories as follows: 1, premenopausal, no HRT; 2, perimenopausal, no HRT; 3, postmenopausal, no HRT; 4, postmenopausal, previous HRT user; 5, postmenopausal, current HRT user.

Fracture assessment at the baseline visit (prevalent fractures) was based on the questionnaire, whereas fracture data obtained at the follow up visit (incident fractures occurring between baseline and follow up) were confirmed by scrutiny of hospital records and x ray reports. Owing to the young age of the study subjects, the number of incident fractures observed in this population was small (nine hip fractures and 15 clinical vertebral fractures). Because of this, we did not analyse the relation between *ESR1* polymorphisms and fractures in this study. All participants gave written informed consent to be included in the study, which was approved by the Grampian research ethics committee.

Bone mineral densitometry measurements

BMD measurements of the left proximal femur (femoral neck) and lumbar spine (L2–L4) were carried out by dual energy x ray absorptiometry using one of two Norland XR26 or XR36 densitometers (Norland Corporation, Fort Atkinson, Wisconsin, USA). Calibration of the machines was undertaken daily, and quality assurance checked by measuring the manufacturer's lumbar spine phantom at daily intervals and

Table 2 Distribution of long range haplotypes defined by the TA repeat, *PvuII* and *XbaI* polymorphisms in the study population

| Long range haplotype | | | Number of alleles (%) |
|----------------------------------|----------------------------|----------|-----------------------|
| (TA) _n repeat allele* | <i>PvuII</i> - <i>XbaI</i> | LRH code | |
| L | PX | I | 185 (3.38) |
| L | Px | II | 157 (2.87) |
| L | pX | III | 18 (0.33) |
| L | px | IV | 2579 (47.17) |
| H | PX | V | 1693 (30.96) |
| H | Px | VI | 505 (9.24) |
| H | pX | VII | 24 (0.44) |
| H | px | VIII | 307 (5.61) |

*The (TA)_n alleles were classified as L if $n \leq 18$, and H if $n > 18$.
LRH, long range haplotype.

a Hologic spine phantom at weekly intervals. The in vivo precision for XR26 was 1.95% for lumbar spine, and 2.3% for femoral neck. Corresponding values for the XR36 were 1.2% for lumbar spine and 2.3% for femoral neck. Comparison between the XR26 and XR36 was made using 50 phantom spine measurements from each machine. The XR36 was consistently found to give slightly higher measurements (2.4%) than the XR26. BMD measurements obtained from the XR36 were therefore corrected to correspond with the XR26 by regression analysis.

Quantitative ultrasound measurements

Quantitative ultrasound (QUS) measurements were carried out using a Hologic Sahara scanner (Hologic Inc, Bedford, Massachusetts, USA). This measures speed of sound (SOS, m/s) and broadband ultrasound attenuation (BUA, dB/MHz), by assessing the "loss" of sound waves as they travel across the calcaneus. This is a dry system where transducers are in direct contact with the skin, and an oil based gel is used as a coupling device. In vitro precision (coefficient of variation) in our hands is less than 1% when examining 10 phantoms.

Genotyping

Genomic DNA was extracted from peripheral blood leucocytes using the Nucleon II DNA extraction kit (Scotlab, Coatbridge, Lanarkshire, UK) according to the manufacturer's instructions. The *PvuII* and *XbaI* polymorphisms are 45 bp apart and located approximately 400 bp upstream of exon 2. Genotypes for the *PvuII* and *XbaI* polymorphisms

Table 3 Relation between bone phenotypes and *ESR1* alleles in the whole study population

| Characteristic | ESR1 polymorphism | | | ESR1 haplotype | |
|-------------------|-------------------|--------------|-------------|----------------|--------------|
| | TA repeat | <i>PvuII</i> | <i>XbaI</i> | <i>px</i> | <i>L-p-x</i> |
| Baseline LS BMD | | | | | |
| Crude | 0.44 | 0.97 | 0.79 | 0.91 | 0.62 |
| Adjusted | 0.47 | 0.98 | 0.73 | 0.99 | 0.71 |
| Baseline FN BMD | | | | | |
| Crude | 0.90 | 0.77 | 0.82 | 0.71 | 1.00 |
| Adjusted | 0.92 | 0.88 | 0.77 | 0.75 | 0.88 |
| Changes in LS BMD | | | | | |
| Crude | 0.82 | 0.17 | 0.72 | 0.36 | 0.62 |
| Adjusted | 0.96 | 0.48 | 0.97 | 0.74 | 0.76 |
| Changes in FN BMD | | | | | |
| Crude | 0.37 | 0.59 | 0.69 | 0.31 | 0.09 |
| Adjusted | 0.34 | 0.27 | 0.48 | 0.15 | 0.06 |
| Calcaneal BUA | | | | | |
| Crude | 0.37 | 0.004 | 0.58 | 0.003 | 0.002 |
| Adjusted | 0.42 | 0.009 | 0.66 | 0.010 | 0.008 |
| Calcaneal SOS | | | | | |
| Crude | 0.45 | 0.08 | 0.99 | 0.07 | 0.012 |
| Adjusted | 0.57 | 0.14 | 0.95 | 0.14 | 0.023 |

Values presented are p values obtained from analysis of variance for crude data and analysis of covariance for the adjusted data. Lumbar spine and femoral neck bone mineral density (BMD) values were adjusted for age, body mass index (BMI), menopausal status, and hormone replacement therapy (HRT) use. Change in BMD values are presented as per cent per year and were adjusted for age, BMI, change in BMI, baseline BMD, menopausal status, and HRT use. Broadband ultrasound attenuation (BUA) and speed of sound (SOS) values were adjusted for age, BMI, BMD, menopausal status, and HRT use. FN, femoral neck; LS, lumbar spine.

were determined by direct DNA sequencing. A 346 bp polymerase chain reaction (PCR) product containing both polymorphic sites was amplified by PCR as described previously.³² PCR products were then cleaned using ExoSAP ITTM kit (USB Corp, Cleveland, Ohio, USA) and sequenced using the DYEnamic ET dye terminator cycle sequencing kit (Amersham Pharmacia Biotech UK, Buckinghamshire, UK). DNA sequencing was carried out in 96-well plates which were run on a MEGABACE-1000 automated DNA sequencer (Amersham Pharmacia Biotech). The genotypes were represented as *Pp* (*PvuII*) and *Xx* (*XbaI*), with upper case letters signifying the absence of, and lower case letters the presence of, the restriction site.

The TA repeat polymorphism of the *ESR1* gene was investigated by PCR using a FAM labelled forward primer (5'-FAM-GACGCATGATATACTTACC-3') and reverse primer (5'-GCAGAATCAAATATCCAGATG-3') in a 25 µl PCR reaction containing: 2.5 µl 10X PCR buffer (Qiagen, Crawley, E Sussex, UK), 2.0 µl of dNTP (10 mM each), 5.0 µl of Q solution, 2.5 µl of oligonucleotide primer mix (5 µM each of forward and reverse primer), 0.125 µl of 5 U/µl *Taq* DNA polymerase (Qiagen), and 50–100 ng genomic DNA. The reaction volume was made up to 25 µl using deionised water. The following thermal cycling conditions were used on a Gene Engine thermocycler (MJ Research Inc, Watertown, Massachusetts, USA): one cycle of (94°C for four minutes, 58°C for 30 seconds, 72°C for 30 seconds) followed by 30 cycles of (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds). The length of PCR product was determined by analysis on a MEGABACE-1000 using the Genetic ProfilerTM software (Amersham Pharmacia Biotech). Genotypes were scored independently by two different individuals. To check for genotyping errors, 50 DNA samples were selected randomly and reanalysed. All genotypes were identical to those obtained from the first round analysis. The genotyping success rate was 94% for *PvuII* and *XbaI* and 96% for TA repeat polymorphism. Our genotyping method for TA repeat was found to estimate systematically one less TA repeat than the method used by researchers who also studied this polymorphism in a cohort of individuals from the

Rotterdam study.³² This was found by independent genotyping of 50 samples from our population carried out by the Rotterdam research group.³²

Statistical analysis

Haplotypes were inferred from genotype data using the PHASE software program.⁴¹ The probability of the *PvuII-XbaI* haplotype phase being correctly inferred was >0.99, and the average probability for the *TA-PvuII-XbaI* haplotype being correctly inferred was 0.987 (95% confidence interval, 0.983 to 0.991). Statistical analyses were carried out using SPSS version 10.0 (SPSS Inc, Chicago, Illinois, USA). Differences in BMD, BUA, and bone loss between the genotype and haplotype groups were tested using analysis of covariance (ANCOVA) to adjust for confounding factors such as age, body mass index (BMI), BMD, menopausal status, and HRT use. For the haplotype analysis, study subjects were coded according to whether they had two copies, one copy, or no copies of the haplotype under investigation. Multiple linear regression analysis was used to test for gene dose effects. Power calculations indicated that the study had >97% power to detect differences in BMD and BUA of 0.1 SD units between genotypes assuming a minor allele frequency of 30%. Probability (p) values of <0.05 were considered significant. We also used a modified Bonferroni correction⁴² to adjust for the number of independent tests carried out, taking into account that the three polymorphisms are in strong linkage disequilibrium and the variables tested are strongly correlated with each other. The corrected threshold of significance was $p < 0.015$. As it has been argued that the Bonferroni correction may be too conservative,⁴³ we present the unadjusted p values in the text.

RESULTS

Characteristics of study population

Table 1 shows relevant demographic characteristics of the study population at the baseline and follow up visits. The average age of the study subjects was 48.5 years at baseline, and at this time 46.3% of women were postmenopausal. The average age at the follow up visit was 54.7 years and the

majority of women were postmenopausal (89.9%). Values for age, BMI, lumbar spine BMD, and femoral neck BMD at the baseline visit were, as expected, significantly different from those at the follow up visit ($p < 0.001$). The average bone loss per year over the 6.2 years follow up was 0.61% and 0.74% at lumbar spine and femoral neck, respectively. Table 1 also shows BMD and QUS values in the study population according to menopausal status and HRT use. At the follow up visit, 59% of the women were either current or previous HRT users. There was a significant association between HRT use and age at menopause, so that subjects who were current HRT users had earlier age at menopause (mean (SD), 44.6 (8.6) year) than those with previous HRT treatment (46.5 (6.6) years) or those without HRT treatment (48.4 (5.7) years) ($p < 0.001$).

Genotype and haplotype frequencies

On DNA sequencing analysis, we were only able to detect the *PvuII* and *XbaI* polymorphisms in our population, even though single nucleotide polymorphisms (SNP) databases suggest the presence of four additional SNPs in the sequenced region. We conclude therefore that these SNPs are absent or very rare in our population. The genotype frequencies for the *PvuII* and *XbaI* polymorphisms were: *PP* = 643 (22.4%), *Pp* = 1383 (48.2%), *pp* = 845 (29.4%), *XX* = 347 (12.1%), *Xx* = 1328 (46.3%), and *xx* = 1193 (41.6%). These were in Hardy-Weinberg equilibrium and are similar to those previously reported in white populations.^{29–30–35} The *PvuII-XbaI* haplotype frequencies were: *PX* = 1960 (34.5%), *Px* = 685 (12.0%), *pX* = 47 (0.8%), and *px* = 2998 (52.7%). There was strong linkage disequilibrium between *PvuII* and *XbaI* polymorphisms ($D' = 0.956$, $p < 0.000001$) and two common haplotypes (*PX* and *px*) accounted for 87.1% of alleles defined by these two polymorphisms, as previously reported in white populations.^{30–31} For the TA repeat polymorphism, we observed 23 different alleles with TA repeat number ranging between 8 and 30 (supplementary figure; see on the JMG website at www.jmedgenet.com/supplemental). This polymorphism showed a bimodal distribution, with two peaks at 15 repeats (31.6% of alleles) and 24 repeats (10.2% of alleles), and a breakpoint at 18 TA repeats as reported previously.^{30–32}

For statistical analysis, TA repeat alleles were classified into two groups on the basis of whether the number of repeats was above the breakpoint ("H alleles"; > 18 repeats) or below ("L alleles"; ≤ 18 repeats). When divided upon this basis, the

TA repeat genotype frequencies were *LL* = 867 (29.5%); *LH* = 1433 (48.7%); and *HH* = 643 (21.8%). Strong linkage disequilibrium was observed between TA repeat and *PvuII-XbaI* polymorphisms, such that low numbers of TA repeats (*L*) were in strong linkage disequilibrium with the "*p*" allele of the *PvuII* polymorphism ($D' = 0.755$; $p < 0.000001$) and the "*x*" allele of the *XbaI* polymorphism ($D' = 0.803$; $p < 0.000001$). Long range haplotypes (LRH) defined by the TA, *PvuII*, and *XbaI* polymorphisms are shown in table 2. All eight possible haplotypes were found, and two common ones—"L-*p-x*" (47.17%) and "H-*P-X*" (30.96%)—accounted for 78.13% of the alleles observed, in keeping with those reported previously in elderly Dutch subjects.³²

Association between *ESR1* alleles and osteoporosis related phenotypes

We found no significant association between any of the *ESR1* polymorphisms or haplotypes and BMD at the lumbar spine or femoral neck at baseline in the whole study population, or with annual rates of bone loss (table 3). Similarly, we found no association between *ESR1* alleles and other confounding variables such as age, BMI, or age at menopause (data not shown).

Analysis of *PvuII-XbaI* haplotypes in relation to calcaneal QUS at follow up showed that BUA values were significantly reduced in subjects carrying the *px* haplotype (mean (SEM) BUA, 75.0 (0.4); $n = 1743$) compared with those without this haplotype (77.1 (0.7); $n = 532$) ($p = 0.005$). The results remained significant after adjusting BUA values for age, BMI, BMD, menopausal status, and HRT use (*px* BUA = 75.0 (0.4); no *px* BUA = 77.2 (0.7)) ($p = 0.006$). A similar trend was observed for calcaneal speed of sound (SOS) but the results were of borderline significance (table 3). There was no significant association between the TA repeat polymorphism and BUA except when the data were analysed as long range haplotype with the intron 1 polymorphisms, when we observed reduced BUA and SOS values in carriers of the *L-p-x* haplotype (BUA = 74.8 (0.4); SOS = 1551.9 (0.8); $n = 1570$) compared with subjects who did not carry this haplotype (BUA = 77.2 (0.7); SOS = 1555.6 (1.3); $n = 618$) ($p = 0.003$ and 0.018, respectively). This result remained statistically significant after adjusting for age, BMI, BMD, menopausal status, and HRT use (*L-p-x* BUA = 74.9 (0.4), no *L-p-x* BUA = 77.0 (0.6); *L-p-x* SOS = 1552.2 (0.8), no *L-p-x* SOS = 1555.4 (1.2)) ($p = 0.005$ and 0.019, respectively).

Table 4 Association between *ESR1* alleles, follow up bone mineral density, and bone loss in postmenopausal women who were not taking hormone replacement therapy

| Characteristic | Number of copies of <i>px</i> haplotype | | | p Value |
|-----------------|---|----------------|----------------|---------|
| | 0 | 1 | 2 | |
| Number of cases | 212 | 433 | 231 | |
| LS BMD | | | | |
| Crude | 0.995 (0.012) | 0.998 (0.008) | 0.967 (0.009) | 0.042 |
| Adjusted | 0.996 (0.011) | 0.997 (0.007) | 0.969 (0.010) | 0.067 |
| FN BMD | | | | |
| Crude | 0.842 (0.009) | 0.833 (0.006) | 0.811 (0.007) | 0.014 |
| Adjusted | 0.842 (0.008) | 0.833 (0.005) | 0.814 (0.007) | 0.019 |
| LS BMD change | | | | |
| Crude | -1.273 (0.072) | -1.348 (0.052) | -1.382 (0.063) | 0.532 |
| Adjusted | -1.273 (0.068) | -1.356 (0.048) | -1.363 (0.066) | 0.549 |
| FN BMD change | | | | |
| Crude | -1.063 (0.069) | -1.231 (0.052) | -1.274 (0.057) | 0.066 |
| Adjusted | -1.051 (0.065) | -1.204 (0.046) | -1.286 (0.062) | 0.030 |

Values are mean (SEM).

Lumbar spine and femoral neck bone mineral density (BMD) values were adjusted for age, body mass index (BMI), and years since menopause; change in BMD values (per cent per year) were adjusted for age, BMI, change in BMI, baseline BMD, and years since menopause.

FN, femoral neck; LS, lumbar spine.

In view of the fact that HRT is known to have a profound influence on bone loss, we investigated annual rates of bone loss in the subgroup of women who were or had become postmenopausal at the second visit and who had not taken HRT (table 1). As expected, these women lost more bone than premenopausal women and HRT users. No difference was observed in distribution of *ESR1* polymorphisms or haplotypes in these three groups (data not shown). A significant association was found between the *px* haplotype and bone loss at the femoral neck in postmenopausal women not taking HRT, however, and this remained significant after adjustment for age, BMI, changes in BMI, baseline BMD, and years since menopause (table 4).

There was clear evidence of a gene dose effect, such that the annual percentage fall in femoral neck BMD increased by 11.8% per copy of the *px* haplotype ($p = 0.009$). When the analysis was repeated using information from the TA repeat polymorphism, the same trends were observed for the *L-p-x* haplotype but the result was not significant ($p = 0.06$). Analysis of BUA in the non-HRT users showed that subjects with two copies or one copy of the *px* haplotype had lower BUA values (73.1 (0.7); $n = 518$) than those without this haplotype (73.8 (1.3); $n = 166$), but this difference was not significant ($p = 0.60$), possibly because the number of individuals with BUA data in the non-HRT user group was small (684) compared to the total study population (2275). In keeping with this, we estimated that a subgroup size of more than 1250 would be required to attain 90% power to detect the difference in BUA observed between the genotype groups in the whole study population (0.14 SD units).

Analysis of *ESR1* polymorphisms in the HRT user group showed a significant association between the *px* haplotype and BUA so that subjects with one or two copies of the *px* haplotype had lower adjusted BUA values (75.3 (0.5); $n = 1043$) than those without this haplotype (78.7 (0.9); $n = 318$) ($p = 0.001$) and no association was found between any *ESR1* genotype or haplotype and BMD or its annual changes in this subgroup. *ESR1* polymorphisms were not associated with any bone related phenotype in the pre- and perimenopausal subgroup.

DISCUSSION

The *ESR1* gene encoding the oestrogen receptor α is a strong candidate for the genetic regulation of bone mass, bone remodelling, and postmenopausal bone loss. However, studies of *ESR1* alleles in relation to BMD have yielded inconsistent results,^{23 27–38} possibly because most studies have been of small sample size and involved subjects of different ages, menopausal status, and ethnic background. In this study, we investigated the relation between *ESR1* polymorphisms and several phenotypes relevant to the pathogenesis of

osteoporotic fractures in a large population based cohort study of white women from Scotland who had been prospectively followed for an average of 6.2 years. While we observed no association between *ESR1* alleles and BMD or bone loss in the whole study population, there was a significant association between the *px* haplotype and rates of bone loss at the femoral neck in postmenopausal women who had not taken HRT. The annual rates of femoral neck bone loss were about 14% higher in subjects who carried one copy of *px* and 22% higher in those carrying two copies compared with individuals who did not carry the *px* haplotype. Reflecting this, we also detected an association between the *px* haplotype and femoral neck BMD in this subgroup of women, such that femoral neck BMD values were 1% lower in *px* heterozygotes and 3% lower in *px* homozygotes at follow up than in subjects who did not carry the *px* allele.

We also found a strong association between the *px* allele and broadband ultrasound attenuation at the calcaneus. This was observed in the whole study population and HRT users, and the association remained significant after adjusting for BMD and confounding factors such as age, BMI, and menopausal status. While the association we observed between calcaneal SOS and *ESR1* polymorphisms was much weaker, previous studies in a large white cohort have shown that BUA is a better predictor of fracture risk than SOS.⁵ The association between BUA and the *px* allele in non-HRT users did not reach statistical significance although this could have reflected the small sample size of this subgroup.

There have been few previous studies of *ESR1* polymorphisms in relation to quantitative ultrasound properties of bone. Those that have been done showed an association between the *ESR1 XbaI* polymorphism and calcaneal quantitative ultrasound (QUS) indices in young white women aged 18 to 35 years³⁷ and in Korean postmenopausal women.³⁸ While neither of these groups carried out haplotype analysis, both studies reported reduced QUS values (stiffness index and BUA) in association with the *x* allele, which is in broad agreement with the findings reported here, where the *px* haplotype was associated with reduced BUA. In another study, no association was observed between the *XbaI* polymorphism and calcaneal QUS in postmenopausal women taking HRT,³⁶ although the sample size in that study was about one fifth as large as the population described here. The present study is the first to investigate haplotypes of all three *ESR1* polymorphisms in relation to BUA and the first to report an association with calcaneal BUA independent of BMD. The association we observed with BUA is of relevance in light of a recent meta-analysis which showed that carriers of the *XX* genotype had a substantially reduced risk of osteoporotic fractures (odds ratio 0.66) when compared with

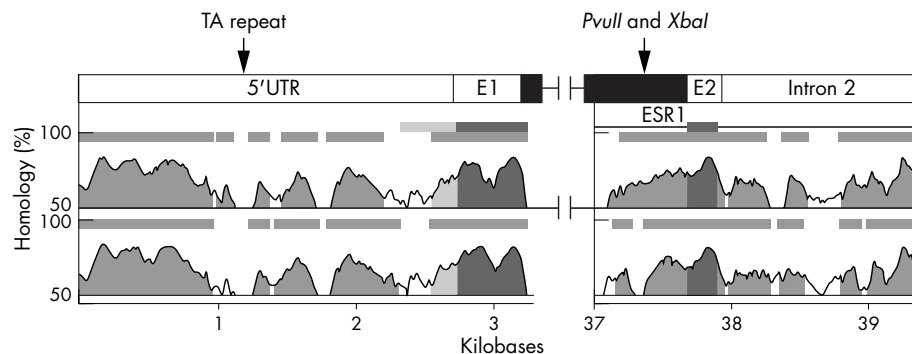


Figure 1 Evolutionarily conserved regions in the human *ESR1* gene compared with the mouse (upper panel) and rat (lower panel) genome. E1 and E2, exons 1 and 2. The first intron spans approximately 34 kb and is shown as a black box (<http://ecrbrowser.dcode.org/>).

xx homozygotes, and that this was out of proportion to the relatively small association between the *XbaI* polymorphism and BMD (approximately 0.15 g/cm²).²⁴ This raises the possibility that *ESR1* polymorphisms might influence fracture risk by an effect on bone quality that is captured by BUA measurements but not by BMD measurements.⁶

In a previous study of older postmenopausal women derived from the same region of the United Kingdom (mean age, 64.4 years), we observed an association between *Px* haplotype and BMD. These results were based on a small sample size ($n = 206$), however, and emphasise the importance of carrying out large scale studies for the genetic dissection of complex traits such as BMD.³¹ The association we observed in the present study between the *Px* haplotype and increased rates of postmenopausal bone loss is in broad agreement with the findings previously reported in a cohort of elderly women from the Rotterdam study, where the *Px* haplotype was associated with reduced lumbar spine BMD and an increased risk of vertebral fracture.³² While these investigators did not investigate allelic associations with bone loss, our data would be consistent with the hypothesis that *ESR1* alleles regulate BMD by affecting bone loss rather than peak bone mass. The fact that the association we observed was restricted to a group of postmenopausal women who had not taken HRT is expected, given the known protective influence of HRT on rates of bone loss. In view of this, we speculate that under conditions of oestrogen sufficiency—such as in premenopausal women and HRT users—the association between *ESR1* polymorphisms and bone turnover may be masked but may become progressively more apparent with increasing age in postmenopausal women who are oestrogen deficient.

The association we observed between *ESR1* polymorphisms and bone loss was stronger for femoral neck than for lumbar spine. Although lumbar spine would be expected to be the more sensitive site to changes in HRT, the associated site may vary between different populations. Previous meta-analysis of *ESR1* polymorphisms showed differences in the skeletal site associated with *ESR1* polymorphisms among the different populations, and when all populations were analysed together the study showed equal effect of *ESR1* alleles on lumbar spine and femoral neck BMD.²⁴

There has previously been controversy over whether the associations between BMD and polymorphisms in the 5' region of the *ESR1* gene are driven by the intron 1 polymorphisms or the TA repeat polymorphism in the *ESR1* promoter. Some investigators have reported that the TA repeat polymorphism is primarily responsible for driving the association with BMD,^{30–32} whereas others, including our own group, have reported that the association is driven by the intron 1 alleles.^{23–31} In the present study we found no significant association between the *ESR1* TA repeat polymorphisms and bone related phenotypes except on long range haplotype analysis, where subjects with a low number of TA repeats who had carried the *Px* haplotype were found to have reduced BUA values. Although this suggests that in our population the association we observed was primarily driven by the *Px* haplotype, it is difficult to be confident about this, given the strong linkage disequilibrium that exists between these polymorphisms.

While *ESR1* is one of the most intensively studied candidate genes for osteoporosis susceptibility, the mechanisms that underlie the associations between bone phenotypes remain unclear. Previous studies have shown that the *PvuII* polymorphism lies within consensus recognition sites for the AP4 and Myb transcription factors.^{31–44} Studies using promoter-reporter assays have shown that the *PvuII* polymorphism influences Myb driven transcription in vitro,⁴⁴ and other studies have shown that both *XbaI* and *PvuII* polymorphisms

also influence reporter gene transcription in vitro.⁴⁵ In this regard, it is of interest that the *PvuII* and *XbaI* polymorphisms are located within a region that is 70–80% conserved in the human, mouse, and rat genomes, whereas the TA repeat polymorphism is not conserved to any significant extent across species (fig 1). While our observations would be consistent with a direct functional effect of the *PvuII* and *XbaI* polymorphisms on *ESR1* gene transcription we cannot exclude the possibility that these polymorphisms are in linkage disequilibrium with causal polymorphisms elsewhere in the *ESR1* gene. Further functional analysis will be required to investigate this possibility.

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Supplementary figure. The *ESR1* TA repeats polymorphism allele distribution in the study population. The figure can be viewed on the JMG website: www.jmedgenet.com/supplemental

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